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Influence of Grape Composition on Red Wine Ester Profile: Comparison between Cabernet Sauvignon and Shiraz Cultivars from Australian Warm Climate

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ABSTRACT: The relationship between grape composition and subsequent red wine ester profile was examined. Cabernet Sauvignon and Shiraz, from the same Australian very warm climate vineyard, were harvested at two different stages of maturity and triplicate wines were vinified. Grape analyses focused on nitrogen and lipid composition by measuring 18 amino acids by HPLC-FLD, 3 polyunsaturated fatty acids, and 6 C6-compounds derived from lipid degradation by GC-MS. Twenty esters and four higher alcohols were analyzed in wines by HS-SPME-GC-MS. Concentrations of the ethyl esters of branched acids were significantly affected by grape maturity, but the variations were inconsistent between cultivars. Small relative variations were observed between wines for ethyl esters of fatty acids, whereas higher alcohol acetates displayed the most obvious differences with concentrations ranging from 1.5- to 26-fold higher in Shiraz than in Cabernet Sauvignon wines regardless of the grape maturity. Grape analyses revealed the variations of wine ester composition might be related to specific grape juice nitrogen composition and lipid metabolism. To the authors' knowledge the present study is the first to investigate varietal differences in the ester profiles of Shiraz and Cabernet Sauvignon wines made with grapes harvested at different maturity stages.

KEYWORDS: *yeast metabolism, nitrogen, lipids, grape berry maturity*

INTRODUCTION

Wine aromatic composition is one of the primary attributes assessed by the wine consumer and is an integral component of the overall wine tasting experience. Wine aromatic compounds are classified within three different groups according to their origin: varietal (primary), fermentative (secondary), and derived from aging (tertiary). Although the tertiary component is primarily important in aged wines, the varietal and fermentative components are crucial for wine aroma quality regardless of wine age.[1] Fermentative aromas are mainly derived from yeast activity and are considered as the wine-like aromatic base common to all wines.[1] The varietal component, which is derived from grape aroma and aromatic precursors, imparts specific aromas depending on the cultivars and vineyard characteristics. Within wine categories produced by a similar winemaking process, varietal aromas are considered to contribute to the unique characters.[2] Therefore, the varietal component regulated by the environment and cultural practices appears to be more related to the concept of wine aromatic typicality[2] than the fermentative component.

Several key markers of varietal aroma have been identified in white cultivars such as Muscat, Sauvignon blanc, and Gewürstraminer.[1] In contrast, the understanding of red wine aroma is less advanced, possibly because of the higher level of complexity of such a medium. A limited number of key varietal markers associated with herbaceous[3] and peppery aromas[4] have been identified in red wines. Conversely, the origin of fruity aroma in red wines is still poorly understood despite the evidence of the existence of fruity attributes (red and black berry) typical of red wines.[5] Several studies have emphasized the potential role of some grape-derived compounds such as C13-norisoprenoids and furanones in the perception of fruity aromas specific to red wines.[6−8] However, the contribution of these markers in red wine aroma seems to be indirect through complex perceptive interactions.[7,8]

Other authors have recently highlighted the potential role of esters in the fruity attributes typical of red wines via similar complex interactions.[7−9] Most wine esters are enzymatically synthesized by yeast during alcoholic fermentation[10] and can also be modulated by the lactic acid bacteria (LAB) during malolactic fermentation (MLF).[11,12] However, the possibility of fermentation-derived volatiles, such as esters, playing an important role in varietal differentiation of wines has not been studied extensively. The subsequent opportunity to utilize these esters as "varietal markers" is therefore novel and contradicts paradigms that have been previously assigned to varietal and fermentative aroma and the understanding of wine aroma perception. A lack of knowledge of "key" aromatic compounds responsible for typical fruity aromas of red wines does not necessarily mean that they do not exist.[9] Conversely, esters are known to significantly contribute to aromas of red and black berry fruits generally used as attributes to describe the specific aromas of red wines.[13] Furthermore, it has been shown that esters such as isoamyl acetate contribute

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significantly to the varietal aromas of Pinotage[14] and Tempranillo.[15]

Although it is accepted that grape berry parameters such as nitrogen status[16] influence the final wine ester profile, the association between grape and wine ester compositions is poorly understood and requires investigation. This study examined the relationship between grape composition and red wine ester profiles by comparing Cabernet Sauvignon and Shiraz at two different stages of maturity, from a warm to very warm and irrigated region of Australia (Riverina, New South Wales). Distinct cultivars were utilized to contrast the ester profiles between varieties.

MATERIALS AND METHODS

Experimental Vineyard and Harvesting. Grapes were sourced from two adjacent commercial vineyard plots, Shiraz and Cabernet Sauvignon, located within the same larger vineyard in Griffith (Riverina, New South Wales, Australia) (34′17′′00 S; 146′02′′00 E). This vine-growing region is classified as very warm according to the Huglin index[17,18] (HI > 3000). Both cultivars were own-rooted, drip irrigated, and trellised to double cordon with an open sprawling canopy and mechanically pruned. The nitrogen management throughout the season was similar for both cultivars and consisted of fertigating 20 kg of nitrogen per hectare per season via small additions from budburst to veraison. The average crop yields of both plots were approximately 15−20 tons per hectare. Both cultivars were harvested sequentially, at two different dates using the evolution of berry sugar accumulation as indicator in conjunction with °Brix.[19] The first harvest for both cultivars occurred at approximately 21 °Brix and was designated as fresh fruit (FF). The second harvest, designated as mature fruit (MF), occurred at 23 °Brix for both cultivars. At each harvest date, 60 kg of grapes per replicate was randomly harvested across the vineyards with an addition of 40 mg/kg of potassium metabisulfite prior to transport to the Charles Sturt University (CSU)/ National Wine and Grape Industry Centre (NWGIC) experimental winery. On arrival, a 100 berry subsample from each replicate was collected and immediately frozen at −20 °C for further analyses.

Winemaking. All replicates were kept separate during the winemaking process. Grapes were destemmed, crushed, and transferred into 100 L stainless steel vats for fermentation. The acidity of each must replicate was adjusted to approximately pH 3.6 using tartaric acid. Fermentation was performed by EC1118 yeast (*Saccharomyces cerevisiae*) and carried out at 25−26 °C with the use of cool rooms. All ferments had identical nutritional supplements, consisting of an addition of 0.3 g/L GoFerm 24 h after yeast inoculation and 0.1 g/L diammonium phosphate (DAP) and 0.25 g/L Fermaid A after a 6 °Brix decrease. This corresponded to total additions of 57 and 15 mg N/L of ammonium and free amino nitrogen, respectively. Malolactic fermentation was carried out using a co-inoculation procedure, with Enoferm alpha LAB (*Oenococcus oeni*) added 2 days post yeast inoculation. Ferments were pressed to 1 bar pressure using a small basket press when the residual sugar decreased to <0.5 g/L. The wine temperature was then maintained at 22 °C until the malic acid concentration decreased to <0.05g/L. The wine was then racked off the gross lees into variable-capacity tanks to prevent ullage and 80 ppm of SO_2 added to achieve levels of 25−30 ppm of free SO_2. Tanks were then moved to a −4 °C cool room for 3 weeks to achieve cold stabilization and racked a further two times during this period for clarification. Heat stability was tested and SO_2 concentrations were adjusted to approximately 30 ppm of free SO_2 to achieve 0.5−0.8 molecular SO_2.Wines were bottled using a four-head filler with CO_2 utilized to prevent oxidation. Bottles were also filled with CO_2 prior to filling and then capped with a Stelvin screwcap.

General Analyses of Grape Maturity, Yeast Available Nitrogen, and Wine Composition. Grape juice samples collected after crushing were analyzed for basic maturity and fermentation parameters. Total soluble solids (TSS), expressed as °Brix, were analyzed with a portable density meter (Anton Paar DMA 35N, Graz, Austria). Titratable acidity (TA) and pH were determined by sodium hydroxide titration to the end point pH 8.2 with an automatic titrator (Metrohm Fully Automated 59 Place Titrando System, Metrohm AG, Herisau, Switzerland). Ammonium, α-amino acids, and acetic acid were determined using commercially available enzymatic tests purposely designed and deployed on an Arena discrete analyzer (ThermoFisher). Yeast assimilable nitrogen (YAN) was calculated from ammonium and free amino nitrogen (FAN) measurements according to a previously published method.[20] Ethanol was measured with an Anton Paar Alcolyser DMA 4500 density meter. Sugar to ethanol conversion rate was calculated as the ratio of potential wine alcohol according to TSS measurements and ethanol level measured in wines.

Ester and Higher Alcohol Analysis in Wines. All of the esters, except ethyl leucate, ethyl acetate, and ethyl phenylacetate, were quantified using a mix of isotopically labeled esters as previously described[21] by employing CDN isotopes (Pointe-Claire, Canada). The final solution used to spike the samples was composed of 20 mg/L [$^2H_5$]-ethyl butyrate, 20 mg/L [$^2H_5$]-ethyl hexanoate, [$^2H_{15}$]-ethyl octanoate, 4 mg/L [$^2H_{23}$]-ethyl decanoate, and 5 mg/L [$^2H_5$]-ethyl cinnamate. Twenty microliters of internal standard mix solution was added to an exact 10 mL volume of wine. A 5 mL sample aliquot was placed into a 20 mL SPME vial previously filled with 3 g of NaCl and diluted with 5 mL of deionized water. The samples were immediately capped and analyzed by HS-SPME-GC-MS as described previously by Antalick et al.[21] with a PDMS-CAR-DVB fiber (Supelco, Bellefonte, PA, USA). Fibers were desorbed into an Agilent 7890 gas chromatograph fitted with a DB-WAXetr capillary column (60 m, 0.25 mm, 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA) using a Gerstel MPX autosampler with a Peltier tray cooler at 4 °C. The GC was coupled to a 5975C mass spectrometer (Agilent Technologies) operating in electron ionization mode using selected ion monitoring (SIM) and scan modes simultaneously. All of the esters, except ethyl leucate, ethyl acetate, and ethyl phenylacetate, were quantified using SIM mode as described elsewhere.[21] *trans*-2-Hexenyl and *cis*-3-hexenyl acetates were quantified with ions 100 and 82, respectively. Ethyl leucate and ethyl phenylacetate were semi-quantitatively analyzed in SIM with ions 87 and 91, respectively. Ethyl acetate and the higher alcohols (propanol, isoamyl alcohol, isobutanol, and phenylethanol) were semiquantitatively measured in scan mode using total ion chromatogram (TIC). Relative concentrations were calculated using [$^2H_5$]-ethyl butyrate for ethyl acetate, propanol, isobutanol, and isoamyl alcohol, [$^2H_5$]-ethyl hexanoate for *trans*-2-hexenyl acetate, *cis*-3-hexenyl acetate, and ethyl leucate, and [$^2H_5$]-ethyl cinnamate for ethyl phenylacetate and phenylethanol.

C6-Compounds Analysis in Grapes. Hexanol, hexanal, *E*-2-hexenol, *E*-2-hexenal, *Z*-3-hexenol, and *Z*-3-hexenal were analyzed in grapes using a method adapted from Loscos et al.[22] Grapes were deseeded in the presence of liquid nitrogen and homogenized with a ball mill TissueLyser (Quiagen, Retsch, Shenzhen, China) for 30 s at 20 Hz. Approximately 500 mg of grape berry powder was weighed into the SPME vial in the presence of liquid nitrogen. Two milliliters of acidic buffer containing 5g/L of tartaric acid and 2 g/L of ascorbic acid with pH adjusted to 3.0 was added to the frozen grape berry powder. Samples were spiked with 15 μL of internal standard containing octan-2-ol and [$^2H_{12}$]-hexanal at concentrations of 68 and 46.5 mg/L, respectively, and 0.5 g of sodium chloride was added to the vial. Vials were randomly positioned on a tray cooler set at 4 °C prior to analysis to limit the influence of sample order on the results due to lipoxygenase activity. Samples were analyzed by HS-SPME-GC-MS with a PDMS-CAR-DVB fiber (Supelco). Fibers were desorbed at 250 °C in splitless mode for 1 min into an Agilent 7890 gas chromatograph fitted with a DB-WAXetr capillary column (60 m, 0.25 mm, 0.25 μm film thickness, J&W Scientific) using a Gerstel MPX autosampler with a Peltier tray cooler at 4 °C. The oven temperature was programmed at 40 °C for 5 min, then raised to 220 °C at 3 °C/min, and then held at that temperature for 30 min. The GC was coupled to a 5975C mass spectrometer (Agilent Technologies) operating in electron ionization mode using SIM and scan modes simultaneously. Quantifying ions

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Table 1. Grapes, Juice, and Wine Analytical Parameters[a,b,c]

	Cabff	Cabmf	Shff	Shmf
harvest date:	March 6, 2013	March 22, 2013	Feb 20, 2013	March 6, 2013
grapes				
berry fresh mass (g)	0.81 ± 0.04b	0.79 ± 0.05b	1.11 ± 0.1a	1.04 ± 0.04a
fresh juice				
TSS (°Brix)	20.9 ± 0.5b	22.7 ± 0.3a	21.1 ± 0.1b	23.0 ± 0.2a
pH	3.55 ± 0.01c	3.69 ± 0.04b	3.63 ± 0.04b	3.98 ± 0.03a
TA (g/L)	4.7 ± 0.15a	4.4 ± 0.15ab	4.2 ± 0.09b	3.1 ± 0.06c
FAN (mg/L as N)	73 ± 2c	85 ± 3b	99 ± 5a	104 ± 2a
ammonium (mg/L as N)	115 ± 1a	90 ± 3b	45 ± 2c	37 ± 2d
YAN (mg/L as N)	175 ± 2a	159 ± 1b	136 ± 6c	135 ± 3c
FAN:Am	0.63 ± 0.01d	0.94 ± 0.06c	2.18 ± 0.05b	2.84 ± 0.08a
wine				
pH	3.60 ± 0.01b	3.71 ± 0.01a	3.60 ± 0.01b	3.74 ± 0.01a
TA (g/L)	5.8 ± 0.0a	5.9 ± 0.2a	5.8 ± 0.0a	5.8 ± 0.0a
ethanol (v/v)	10.7 ± 0.2c	11.6 ± 0.3b	11.5 ± 0.1b	12.4 ± 0.0a
acetic acid (g/L)	0.29 ± 0.02b	0.27 ± 0.02b	0.36 ± 0.02a	0.21 ± 0.01c
sugar:ethanol (%)	88.7 ± 2.8bc	86.3 ± 1.5c	93.3 ± 0.6a	91.0 ± 1.0b

[a]Cabff, Cabernet Sauvignon fresh fruit; Cabmf, Cabernet Sauvignon mature fruit; Shff, Shiraz fresh fruit; Shmf, Shiraz mature fruit. [b]One-way ANOVA was used to compare data. Means followed by different letters in a row are significant at $p \leq 0.05$ (Fischer's LSD). All quoted uncertainty is the standard deviation of three replicates per treatment. [c]TSS, total soluble solids; TA, titratable acidity; FAN, free amino nitrogen; YAN, yeast assimilable nitrogen; sugar:ethanol, sugar to ethanol conversion rate; FAN:Am, ratio of free amino nitrogen (mg/L as N) to ammonium concentration (mg/L as N).

chosen for the internal standards were 45 for octan-2-ol and 92 for [$^2H_{12}$]-hexanal. C6-compounds were measured in SIM mode in a semiquantitative way using peak to area ratios and octan-2-ol and [$^2H_{12}$]-hexanal as internal standards for alcohols and for aldehydes, respectively. Compound identity was confirmed by checking the mass spectra in scan mode performed simultaneously to SIM mode and comparing the retention times with those available in the literature. Quantifying ions chosen were 56 for hexanal and hexanol, 83 for *E*-2-hexenal and *Z*-3-hexenal, 57 for *E*-2-hexenol, and 67 for *Z*-3-hexenol.

Lipid Analysis in Grapes. Oleic, linoleic, and linolenic acids were analyzed by semiquantification according to a method adapted from Roufet et al.[23] A 1 g sample of frozen grape powder prepared as previously described was weighed into a 20 mL glass vial and immediately extracted to limit lipid oxidation. Semiquantitative analysis was performed by adding 1 mL of 1 g/L decanoic acid solution as internal standard to samples immediately prior to extraction. The amount of decanoic acid added was approximately 10000 times higher than the initial concentration of this compound present in grape berry samples. Therefore, the initial concentration of decanoic acid present in grapes did not interfere with lipid quantification. Lipid extraction was performed three times for 5 min by stirring in 10 mL, followed by two 5 mL mixtures of chloroform/methanol (2:1). The chloroform phases were collected and pooled. Lipids were thereafter methylated to be analyzed by GC-MS, stirring the chloroform phase with 5 mL of a 3% methanolic solution of HCl for 1 h. The organic phase was washed twice with deionized water and collected for drying over ammonium sulfate. Organic extracts were concentrated to 2 mL by evaporation under low pressure before being injected in GC-MS. A 1 μL sample volume of extract was injected at 250 °C in split mode (ratio 10:1) into the same chromatograph and column as previously described. The oven temperature was programmed at 40 °C, then immediately raised to 220 °C at 8 °C/min, and then held at that temperature for 57.5 min. The mass spectrometer was operating in electron ionization mode using SIM and scan modes simultaneously. Methyl esters of lipids were analyzed in SIM mode in a semiquantitative manner using peak to area ratios and decanoic acid as an internal standard. Quantifying ions chosen were 74 for methyl decanoate, 55 for methyl oleate, 67 for methyl linoleate, and 79 for methyl linolenate. The identity of compounds was confirmed by checking the mass spectra in scan mode performed simultaneously to SIM mode and comparing the retention times with the retention indices available in the literature. Qualification ions were used in SIM mode to avoid any integration mistake (87 for methyl decanoate, 264 for methyl oleate, 294 for methyl linolenate, and 292 for methyl linolenate).

Amino Acid Analysis in Grapes. The extraction protocol for amino acids from frozen grape berry powder was adopted from a previously published method.[24] Grape berry powder sample weight was reduced from 2.5 (±0.1) to 0.1 (±0.0005) g. To maintain the ratio of grape powder/extraction medium 1:1 (w/v),[25] 100 μL of a water/methanol mixture (20:80) was added to the samples. The samples were then vigorously vortexed (MS 3 basic, IKA, Selangor, Malaysia) for 1 min and sonicated at room temperature for 15 min to complete the extraction. Samples were then centrifuged for 10 min at 12000*g*, and the supernatant was collected and placed in a new Eppendorf tube for the derivatization process as described elsewhere.[25] Briefly, grape extracts were diluted 1:20 (v/v) with 0.25 M borate buffer (pH 8.5). The amino groups were derivatized with 9-fluoroenyl-ethyl chloroformate, and an internal standard of 13.1 mg/L L-hydroxyproline was used. Amino acids were analyzed by high-performance liquid chromatography (HPLC) using a Waters 600 controller (Milford, MA, USA) connected to an autosampler (Waters 717 plus). Separation of amino acids was achieved with a reverse phase column (Thermo 5 μm ODS hypersil, Waltham, MA, USA) and quantification with a fluorescence detector (Waters 2475). The column temperature was increased to 45 °C to separate γ-aminobutyric acid and proline. Quantification was performed as previously reported.[24]

Statistical Analysis. One-way and two-way analyses of variance (ANOVA) for the variables cultivar and harvest date were performed on the chemical data using Statistica, version 12 (StatSoft, Tulsa, OK, USA), and the means were separated using Stats-Fisher's LSD test (different letters account for significant differences at $p \leq 0.05$).

RESULTS AND DISCUSSION

Description of Grape Juice Parameters. In the present study both cultivars were harvested at very similar TSS contents (Table 1); therefore, it is unlikely that differences observed in wine ester profile between cultivars for a given maturity stage originated from yeast sugar metabolism. The FF harvest was performed at approximately 21 °Brix, whereas grapes were harvested at around 23 °Brix for the MF harvest (Table 1).

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Table 2. Mean Concentrations of Esters for Each Group of Wines[a]

	concentration[b] (μg/L)				*p* value[c]		
compound	Cabff	Cabmf	Shff	Shmf	cultivar	harvest stage	cultivar × harvest stage
ethyl esters of straight-chain fatty acids							
ethyl propionate	157 ± 5.5a	147 ± 9.3a	151 ± 9.5a	143 ± 8.8a	0.283	0.108	0.824
ethyl butyrate	119 ± 3.7bc	109 ± 5.6c	120 ± 3b	133 ± 8.2a	**0.004**	0.604	**0.008**
ethyl hexanoate	508 ± 18a	434 ± 15b	522 ± 2.2a	549 ± 22a	**<0.001**	**0.035**	**<0.001**
ethyl octanoate	510 ± 30a	451 ± 24b	522 ± 4.3a	571 ± 45a	**0.005**	0.776	**0.012**
ethyl decanoate	124 ± 12ab	105 ± 5.5b	135 ± 12a	138 ± 17a	**0.016**	0.319	0.171
ethyl dodecanoate	15.5 ± 2.6ab	11.9 ± 1.7b	19.5 ± 2.1a	18.5 ± 2.6a	**0.004**	0.125	0.367
higher alcohol acetates							
propyl acetate	31.5 ± 1.3ab	31.8 ± 1.1ab	28.8 ± 2.3b	33.9 ± 1.6a	0.763	**0.018**	**0.033**
isobutyl acetate	18.1 ± 2.1d	21.4 ± 0.99c	34.9 ± 1.4a	28.9 ± 2.6b	**<0.001**	0.261	**0.003**
isoamyl acetate	473 ± 49c	596 ± 39b	1389 ± 34a	1294 ± 123a	**<0.001**	0.737	**0.039**
hexyl acetate	5.6 ± 0.5b	4.9 ± 0.2b	48.2 ± 1.7a	46.4 ± 0.04a	**<0.001**	**0.049**	0.305
trans-2-hexenyl acetate	0.27 ± 0.02b	0.24 ± 0.03b	0.91 ± 0.06a	0.87 ± 0.09a	**<0.001**	0.283	0.834
cis-3-hexenyl acetate	0.021 ± 0.03c	0.013 ± 0.02d	0.47 ± 0.03a	0.31 ± 0.03b	**<0.001**	**<0.001**	**<0.001**
phenylethyl acetate	54 ± 7.5d	71.5 ± 6c	95 ± 10.6b	119 ± 7.8a	**<0.001**	**0.002**	0.508
ethyl esters of branched acid							
ethyl isobutyrate	30 ± 2.7b	43.5 ± 7.3a	54.7 ± 3.6a	34.4 ± 2.6b	**0.017**	**0.023**	**<0.001**
ethyl 2-methylbutyrate	10 ± 0.5b	14.2 ± 1.6a	10.8 ± 0.3b	7.6 ± 0.4c	**<0.001**	0.318	**<0.001**
ethyl isovalerate	11.8 ± 0.5b	16.6 ± 2.1a	14.8 ± 0.37a	10.2 ± 0.5c	**0.037**	0.884	**<0.001**
ethyl leucate[d]	0.008 ± 0.001b	0.010 ± 0.001a	0.009 ± 0a	0.006 ± 0.001c	0.068	0.392	**0.002**
ethyl phenylacetate[d]	1.06 ± 0.19a	1.32 ± 0.08a	0.73 ± 0.06b	0.6 ± 0.04c	**<0.001**	0.356	**0.015**
miscellaneous compounds							
ethyl cinnamate	2.23 ± 0.5a	2.36 ± 0.39a	1.31 ± 0.25b	1.31 ± 0.2b	**0.001**	0.768	0.768
ethyl acetate[d]	2.2 ± 0.1a	2.3 ± 0.1a	2.2 ± 0.1a	2.2 ± 0.2a	0.477	0.404	0.124
propanol[d]	0.45 ± 0.02a	0.47 ± 0.15a	0.34 ± 0.02b	0.44 ± 0.05a	**0.004**	**0.004**	**0.037**
isobutanol[d]	0.13 ± 0.04a	0.16 ± 0.05a	0.16 ± 0.05a	0.14 ± 0.03a	0.889	0.854	0.289
isoamyl alcohol[d]	21.7 ± 1.1b	25.5 ± 1.2a	22.9 ± 0.18b	22.3 ± 1.4b	0.133	**0.031**	**0.007**
phenylethanol[d]	189 ± 29.6a	209 ± 21.3a	138 ± 6.7b	146 ± 10.4b	**<0.001**	0.227	0.559

[a]Cabff, Cabernet Sauvignon fresh fruit; Cabmf, Cabernet Sauvignon mature fruit; Shff, Shiraz fresh fruit; Shmf, Shiraz mature fruit. [b]Two-way ANOVA was used to compare data. Means expressed in μg/L followed by different letters in a row are significant at $p \leq 0.05$ (Fischer's LSD). All quoted uncertainty is the standard deviation of three replicates of one treatment. [c]Significance of two-way ANOVA for cultivar, harvest date, and interaction cultivar × harvest date. Bold numbers indicate significant differences. [d]Compound measured by semiquantification; relative peak areas (compound:internal standard areas ratio) were used to calculate means and standard deviation.

However, the relationship between potential variations in esters during ripening and yeast sugar metabolism is difficult to establish. Yeast ester metabolism depends not only on yeast sugar metabolism,[11] and the relationship between grape berry sugar accumulation and aromatic maturity is complicated, often indirect, and compound specific.[26]

Higher pH and lower TA values were measured for MF stage compared to FF and for Shiraz compared to Cabernet Sauvignon juice (Table 1), but these differences were reduced before fermentation by the addition of tartaric acid. Consequently, TA concentrations were similar in all of the wines, whereas wine pH values were similar between cultivars, remaining higher in the MF stage in comparison to FF (Table 1).

The general nitrogen composition significantly differed between cultivars and, to a lesser extent, maturity stages. Cabernet Sauvignon grapes exhibited higher values of YAN than Shiraz mainly due to higher concentrations of ammonium regardless of nutrient addition (Table 1). In contrast, Shiraz grape juices showed higher concentrations of free amino nitrogen (FAN). Therefore, FAN:ammonium ratios were 3–3.5-fold higher in Shiraz than Cabernet Sauvignon grape juices (Table 1). These values decreased from 1.8- to 2.1-fold higher in Shiraz than Cabernet Sauvignon when the nutrient additions were taken into consideration. The comparison of relative grape juice nitrogen composition between Shiraz and Cabernet Sauvignon has not been previously reported in the literature. In addition, ammonium concentrations significantly decreased during ripening for both varieties, whereas FAN increased over maturation only in Cabernet Sauvignon (Table 1). The decrease of ammonium and the lack of consistency for increasing FAN over grape ripening have both been extensively reported in the literature.[16]

Varietal and Maturity Effect on Esters Concentrations. Quantified esters were grouped according to their origin into three groups: ethyl esters of fatty acids (EEFAs), ethyl esters of branched acids (EEBAs), and higher alcohol acetates (HAAs) (Table 2). A two-way ANOVA was used to assess the effect of cultivar, maturity (harvest date), and cultivar × maturity interactions on esters and amino acid variations. Interestingly, 80% of esters measured in this study were influenced by a varietal effect, whereas only 30% were influenced by grape maturity (Table 2). Similar results were found for most of the amino acids (Table 3).

Ethyl Esters of Fatty Acids. Overall, similar ranges of EEFA concentrations were measured in Cabernet Sauvignon and Shiraz wines (Table 2). However, some variations in EEFAs were observed between wines, and interestingly they were more related to variety than maturity (Table 2). Indeed, five of six EEFAs were influenced by cultivar, whereas only ethyl

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Table 3. Mean Concentrations of Amino Acids with Their Standard Deviations[a]

	concentration[b] (μg/g of berry)				p value[c]		
compound	Cabff	Cabmf	Shff	Shmf	cultivar	harvest stage	cultivar × harvest stage
yeast-preferred amino acids							
aspartic acid	18.7 ± 0.9b	14.7 ± 3.2c	25.1 ± 2.9a	18.9 ± 0.4b	**0.003**	**0.004**	0.409
glutamic acid[d]	0.022 ± 0.001bc	0.013 ± 0.002c	0.034 ± 0.02a	0.027 ± 0.004b	**<0.001**	**<0.001**	0.183
asparagine	2.7 ± 0.4bc	1.7 ± 0.5c	3.6 ± 0.9ab	4.7 ± 0.9a	**0.002**	0.969	**0.030**
glutamine	58 ± 12.6bc	86.9 ± 14.7a	51.3 ± 2.6c	66.7 ± 8.7b	0.059	**0.007**	0.304
serine	44.0 ± 3.3b	43.6 ± 4.0b	43.9 ± 1.7b	52.4 ± 3.9a	0.055	0.068	**0.047**
alanine	42.5 ± 6.3b	43.9 ± 4.6b	52.6 ± 7.8ab	56.1 ± 4.9a	**0.012**	0.493	0.773
arginine	371 ± 38b	336 ± 73b	806 ± 47a	925 ± 121a	**<0.001**	0.373	0.119
branched amino acids							
valine	31.8 ± 4.1b	42.5 ± 5.3a	28.2 ± 0.9b	39.7 ± 3.0a	0.172	**<0.001**	0.844
isoleucine	13.4 ± 1.6b	22.4 ± 2.6a	11.9 ± 1.9b	20.5 ± 4.9a	0.353	**<0.001**	0.902
leucine	25.8 ± 3.3b	33.6 ± 2.7a	23.8 ± 1.7b	36.0 ± 4.1a	0.914	**<0.001**	0.255
phenylalanine	12.3 ± 0.7a	7.8 ± 0.6c	9.8 ± 1.0b	10.4 ± 2.2ab	0.967	**0.030**	**0.008**
other amino acids							
proline	2710 ± 414b	3498 ± 118a	318 ± 13d	556 ± 89c	**<0.001**	**0.004**	0.062
hystidine	3.5 ± 0.4b	4.1 ± 0.2a	2.9 ± 0.2c	3.4 ± 0.7bc	**0.028**	0.052	0.849
glycine	28.8 ± 4.5a	29.8 ± 5.8a	7.6 ± 0.5b	6.8 ± 3.1b	**<0.001**	0.946	0.699
threonine	37.4 ± 2.7b	39.5 ± 7.2b	58.4 ± 3.0a	61.5 ± 6.2a	**<0.001**	0.405	0.867
γ-aminobutyric acid	108 ± 9.5c	114 ± 10.2bc	122 ± 3.0b	155 ± 29a	**0.0187**	0.065	0.175
methionine	11.5 ± 1.6b	18.4 ± 5.2a	3.5 ± 0.2d	5.5 ± 0.9c	**<0.001**	**0.024**	0.161
lysine	5.3 ± 0.5b	5.7 ± 0.5a	9.2 ± 0.9a	11.1 ± 3.2a	**0.003**	0.327	0.533

[a]Cabff, Cabernet Sauvignon fresh fruit; Cabmf, Cabernet Sauvignon mature fruit; Shff, Shiraz fresh fruit; Shmf, Shiraz mature fruit. [b]Two-way ANOVA was used to compare data. Means expressed in μg/g of berry weight followed by different letters in a row are significant at $p \leq 0.05$ (Fischer's LSD). All quoted uncertainty is the standard deviation of three replicates of one treatment. [c]Significance of two-way ANOVA for cultivar, harvest date, and interaction cultivar × harvest date. Bold numbers indicate significant differences. [d]Compound measured by semiquantification, relative peak area (compound:internal standard areas ratio) were used to calculate means and standard deviation.

hexanoate varied according to maturity (Table 2). Similar results have been recently reported in Australian Riesling;[26] however, the differences in EEFA concentrations between Cabernet Sauvignon and Shiraz wines were significant only for the MF stage (Table 2). EEFA concentrations decreased in Cabernet Sauvignon wines during maturation, particularly for ethyl hexanoate and octanoate. These two esters were subject to interactions between cultivar and harvest stage effect (Table 2). On the other hand, EEFA concentrations in Shiraz wines were steady over maturation (Table 2). Increases of EEFAs concentrations during grape ripening have been recently reported in Australian Cabernet Sauvignon.[27] The contrast observed between the different studies suggests that the link between grape composition and yeast EEFA metabolism is complex. EEFAs are generally synthesized by yeast during alcoholic fermentation, and their levels in wines seem to largely depend on winemaking conditions such as fermentation temperature, aeration, skin contact, and yeast strain.[11,28] In the present study these type of parameters (temperature, yeast strain, time of skin contact, punching down frequency) were standardized by the winemaking protocol. In parallel, the nitrogen composition of grape juice seems to influence EEFA synthesis to a lesser extent,[29] and the relative variations observed for this group of esters were the smallest of all the wine volatiles analyzed in this study (Table 2).

Ethyl Esters of Branched Acids. EEBA variations were largely driven by interactions between grape maturity and the choice of the cultivar (Table 2). In the present study Cabernet Sauvignon and Shiraz displayed opposite trends during grape maturation (Table 2). Although grape maturation favored higher concentrations of EEBAs in Cabernet Sauvignon, it significantly reduced EEBA concentrations in Shiraz wines (Table 2). At FF stage the concentrations of ethyl isobutyrate, isovalerate, and leucate were higher in Shiraz wines, whereas the concentrations of these esters and ethyl 2-methylbutyrate were higher in Cabernet Sauvignon wines at MF stage (Table 2). These variations were particularly evident for ethyl isobutyrate, with concentrations of 30 and 54.7 μg/L in Shiraz and Cabernet Sauvignon, respectively, at FF stage, representing a 1.8 factor difference.

EEBAs are primarily synthesized during wine aging by esterification between branched acids and ethanol.[28,30] However, a small proportion is also biosynthesized by yeast during alcoholic fermentation through branched amino acid metabolism.[31] Consequently, branched amino acid juice composition significantly affects EEBA concentrations and their corresponding acids found in wines. In the present study the concentrations of branched amino acids measured in grapes were predominantly influenced by maturity without specific varietal effect (Table 3). The similar concentrations of branched amino acids measured in Cabernet Sauvignon and Shiraz grapes increased with grape maturity. The variations measured during ripening in Cabernet Sauvignon grapes may be linked to the increase of EEBA concentrations observed in the corresponding wines. Prior observations support the trend of increased EEBAs during grape maturation in Cabernet Sauvignon.[27] On the other hand, the decreases of EEBA concentrations with grape maturity observed in Shiraz wines (Table 2) did not reflect the variations of branched amino acids contents in the corresponding grape berries (Table 3). In that case, EEBA variations could be potentially linked to yeast redox metabolism as suggested by previous studies.[28,31] This hypothesis is supported by the similar variations observed in Shiraz wines for acetic acid and the highest variations measured

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for ethyl isobutyrate (Tables 1 and 3). Acetic acid production during alcoholic fermentation is known to depend on yeast redox metabolism,[32] and it has been recently suggested that isobutyric acid could also be a specific marker of yeast redox metabolism.[28,33] However, such speculations will need to be studied in the future using appropriate investigations.

Interestingly, ethyl phenylacetate, ethyl cinnamate, and 2-phenylethanol concentrations were significantly higher in Cabernet Sauvignon wines irrespective of the grape maturity stage (Table 2). Weldegergis et al.[34] reported that Cabernet Sauvignon wines exhibited significantly higher concentrations in 2-phenylethanol than Shiraz wines. Other authors suggested that benzene derivatives could be considered as markers of ripe Cabernet Sauvignon grapes.[35] Ethyl phenylacetate, cinnamate, and 2-phenylethanol are derived from phenyalanine metabolism, originating from yeast, grapes, and yeast and grapes, respectively.[28,35] In the present study, no trend between these compounds in wines and the phenylalanine concentrations of the corresponding grapes are evident (Table 3). The variations measured in wines for ethyl phenylacetate, cinnamate, and 2-phenylethanol might therefore reflect differences of phenylalanine metabolism between Cabernet Sauvignon and Shiraz varieties.

Higher Alcohol Acetates. The most important varietal effect in regard to the relative variations of ester concentrations was observed for HAAs. Conversely, maturity levels exhibited a lower effect on this group of esters (Table 2). Shiraz wines, irrespective of maturity stage, displayed significantly higher concentrations of all HAAs except propyl acetate in comparison to Cabernet Sauvignon wines (Table 2). Two groups of HAAs were influenced by measured parameters: acetates of higher alcohols from yeast nitrogen and sugar metabolism and C6-acetates from grape lipid degradation. Isobutyl, isoamyl, and 2-phenylethyl acetates, classified in the first group, were at concentrations from 1.5- to 3-fold higher in Shiraz compared to Cabernet Sauvignon wines (Table 2). These esters were also influenced by harvest date; however, the effect was ester specific. In Shiraz wines 2-phenylethyl acetate concentration increased with maturity, whereas concentrations of isoamyl and isobutyl acetates decreased slightly. On the other hand, the concentrations of these acetates increased with grape maturity in Cabernet Sauvignon wines. These results are supported by previous observations made in Cabernet Sauvignon wines,[27] whereas there is no previous reported information with regard to Shiraz. Furthermore, important varietal differences were also noted for C6-acetates as hexyl, *cis*-3-hexenyl, and *trans*-2-hexenyl acetates. Concentrations of hexyl and *cis*-3-hexenyl acetates were 9- and 22-fold higher, respectively, in Shiraz than in Cabernet Sauvignon wines. Such quantitative differences in acetate esters, particularly for isoamyl and hexyl acetates, may alter the aromatic profile of red wines.[9]

Investigations on the Origin of Varietal Differences in Higher Alcohol Acetate Composition. Higher alcohol acetates were the group of esters most influenced by variety. Only propyl acetate exhibited variations between harvest dates and cultivars that were well correlated to the variation of the corresponding higher alcohol (Table 2). Other acetates derived from amino acid metabolism, such as isobutyl, isoamyl, and 2-phenylethyl acetate, were quantified at significantly higher concentrations in Shiraz than in Cabernet Sauvignon wines (Table 2). Unlike propyl acetate, no specific relationship could be established between the levels of higher alcohols (isobutanol, isoamyl alcohol, 2-phenylethanol) and the corresponding acetates measured in wines (Table 2). Higher alcohol acetates are mainly synthesized during alcoholic fermentation by yeast from acetyl-CoA and higher alcohols through alcohol acetyltransferase activities.[11] Even though higher alcohol availability strongly influences HAA production, the limiting factor for their synthesis is the expression of alcohol acetyltransferase activities.[11] Oxygen, lipids, and nitrogen are the main nutrients found in must, which are known to particularly regulate alcohol acetyltransferase.[11] It could be reasonably expected that oxygen levels in the grape musts prior to fermentation were similar for all wines due to the standardization of the winemaking protocol. Moreover, unsaturated fatty acids, known to repress alcohol acetyltransferase expression, were higher in Shiraz grapes, whereas Shiraz wines exhibited higher concentrations of HAAs (Figure 1;

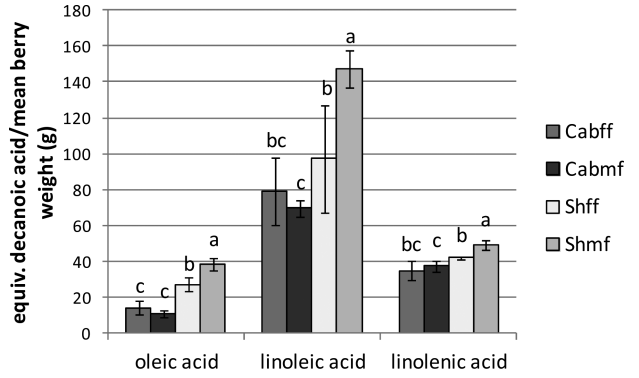

Figure 1. Mean ± SD of unsaturated fatty acids in grape berries expressed as decanoic acid equivalent per gram of berry weight. One-way ANOVA was used to compare data. Different letters above bars indicate significance at $p \leq 0.05$ (Fischer's LSD). All quoted uncertainty is the standard deviation of three replicates per treatment. Cabff, Cabernet Sauvignon fresh fruit; Cabmf, Cabernet Sauvignon mature fruit; Shff, Shiraz fresh fruit; Shmf, Shiraz mature fruit.

Table 2). This contrast indicates that it is unlikely that cultivar differences observed for HAAs between Shiraz and Cabernet Sauvignon wines originated from alcohol acetyltransferase repression due to lipid concentrations. The influence of grape lipids on HAA production in Shiraz wines by boosting cellular growth and general yeast metabolism[36,37] cannot, however, be fully excluded.

Yeast assimilable nitrogen (YAN) is the most common analytical parameter used to assess grape juice nitrogen status. In general, higher YAN concentration favors higher HAA production.[38,39] Must YAN levels could be considered as low to moderate[16] for both cultivars, with slightly higher values in Cabernet Sauvignon compared to Shiraz (Table 1). Consequently, no direct link between YAN and HAA production could be established in the present study. However, YAN composition differed greatly between both varieties with a higher ratio FAN:ammonium measured in Shiraz musts regardless of nutrient addition. This is due to higher FAN and lower ammonium concentrations in Shiraz compared to Cabernet Sauvignon (Table 1).

On the other hand, some of the amino acids assimilable by yeast that contribute to FAN have been classified as "preferred" in regard to their importance for yeast metabolism.[40] Interestingly, Shiraz grapes consistently possessed higher concentrations of "preferred" amino acids than Cabernet Sauvignon (Table 3). High amino nitrogen media may

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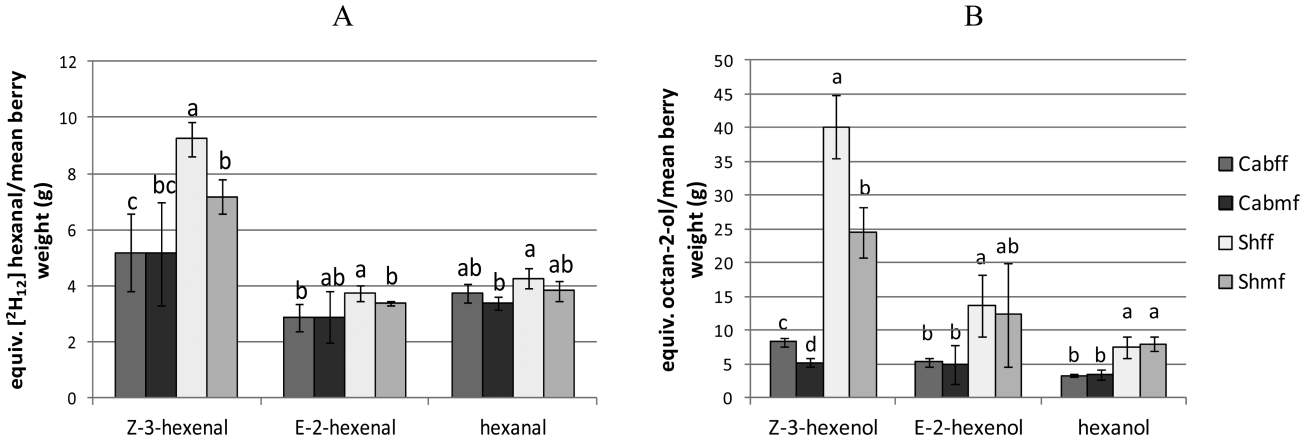

Figure 2. Mean ± SD of C6-aldehydes (A) and C6-alcohols (B) in grape berries expressed as equivalent [$^2H_{12}$]-hexanal and octan-2-ol per gram of berry weight. One-way ANOVA was used to compare data. Different letters above bars indicate significance at $p \leq 0.05$ (Fischer's LSD). All quoted uncertainty is the standard deviation of three replicates per treatment. Cabff, Cabernet Sauvignon fresh fruit; Cabmf, Cabernet Sauvignon mature fruit; Shff, Shiraz fresh fruit; Shmf, Shiraz mature fruit.

stimulate alcohol acetyltransferase expression and, consequently, HAA synthesis by yeast.[41] Furthermore, alcohol acetyltransferase expression has been reported to be particularly important during the early stage of the fermentation.[42] Amino acid uptake by yeast is also known to be maximal during the early phase of alcoholic fermentation as ethanol inhibits their transfer from the external medium into yeast cell.[43] In parallel, ammonium is the first source of nitrogen assimilated by yeast.[16] Therefore, a low ratio of FAN:ammonium in must may down-regulate yeast metabolic activity due to the lower assimilation of amino acids during the early stages of alcoholic fermentation.[43] A relationship between grape nitrogen composition and the varietal differences for HAAs in wines is suggested. Higher levels of FAN and FAN:ammonium ratio found in Shiraz grapes may influence the yeast metabolism that favors alcohol acetyltransferase expression and consequently HAA synthesis. Some cultivars rich in amino nitrogen such as Pinotage are known to exhibit specific esters profile with high concentrations of HAAs.[14] Sugar to ethanol conversion rates were also higher in Shiraz (Table 1). Glycolytic enzymes involved in the conversion of sugar to ethanol are known to be especially influenced by FAN:ammonium balance.[43] This helps explain the differences in HAAs between varieties found in this study and suggests an important relationship between grape nitrogen composition and yeast metabolism regardless of the nitrogen supplementation in the winery. Further studies linking grape nitrogen composition and HAA production by yeast at microbiological and transcriptomic levels are therefore warranted. The influence of other grape metabolites on yeast ester metabolism also requires further investigation.

The varietal differences between Shiraz and Cabernet Sauvignon were the most important for C6-acetates. These esters are derived from the acetylation of C6-alcohols originating from lipid oxidation in grape berries throughout ripening and during crushing.[35,44] Surprisingly, in the present study Shiraz grapes exhibited much higher concentrations of C6-alcohols and C6-aldehydes, particularly *Z*-3-hexenol and *Z*-3-hexenal, than Cabernet Sauvignon (Figure2). Therefore, in this case the stimulation of acetate synthesis in Shiraz wines due to nitrogen composition was accentuated by a higher synthesis of C6-compounds in Shiraz grapes. Interestingly, the levels of linoleic and linolenic acid, from which C6-aldehydes and alcohols are derived, were similar at FF stage between Shiraz and Cabernet Sauvignon grapes (Figure 1), whereas C6-compound contents were significantly higher in Shiraz compared to Cabernet Sauvignon grapes (Figure 2). This result suggests that in the vineyards studied, some enzymes involved in the lipoxygenase pathway may have been more active in Shiraz in comparison to Cabernet Sauvignon, at least for the early stage of grape maturation. For the MF stage, unsaturated fatty acids were measured at higher levels in Shiraz compared to Cabernet Sauvignon grapes (Figure 1), causing the origin of varietal differences of C6-compounds in grapes to be less clear. Further studies that investigate potential differences in the lipoxygenase pathway between Shiraz and Cabernet Sauvignon would be of interest.

This study has demonstrated that red wine ester profiles could be strongly influenced by grape composition and, in particular, grape nitrogen and lipid metabolism. New perspectives for a more detailed investigation to determine the impact of nitrogen and lipid interactions on yeast ester metabolism have been proposed. The present study is the first to investigate varietal differences in ester profiles of Shiraz and Cabernet Sauvignon wines made with grapes harvested from commercial vineyards at different maturity stages. This work was performed in a very warm climatic region of Australia on irrigated vines. Consequently, the variations in ester profiles between Shiraz and Cabernet Sauvignon wines observed in this study may reflect varietal differences in grape composition arising from different responses to abiotic factors and potential stress or constraints (temperature, heat waves, water constraint pre- and post-veraison, and light quality and quantity at the canopy and fruit zone levels) as it has been recently reported in Sauvignon blanc wines.[45] Further investigations will be needed to determine if the varietal differences observed in this study were specific to the site chosen or if they can be extrapolated to a wider scale.

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